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TOLUENE DOSE-RESPONSE AND PRELIMINARY STUDY OF PROTEOMICS FOR NEURONAL CELL LINES

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14. ABSTRACT
Toluene, a jet fuel component, is a potential contaminant in aircraft. Effects of toluene exposure include headache or fatigue. The objective of this study was to perform a preliminary investigation of the cellular and molecular effects of toluene on murine cell lines including Cath.a, C8-B4, CLU199, and HT22. The study tests two methods: direct dosing of toluene in the media and vapor exposure in glass chambers. To better define the complex signaling response to toluene, the two exposure models are characterized by cell viability assays and Tandem Mass Tags (TMT) liquid chromatography mass spectrometry (LC-MS/MS) quantitative proteomics proteome pathway analysis. Cell viability in response to direct dosing in a vehicle demonstrated a dose-dependent decrease in cell viability for all cell lines. Cytoscape 3.1.0 Reactome FI pathway analysis of Cath.a cells following direct dosing with 0.2 and 2 mM toluene revealed a dose-dependent toxicity increase of modulated protein nodes related to oxidative stress such as energy reserve metabolism, cell-death signaling, cytoskeletal rearrangement, DNA damage response, targeted protein degradation and apoptosis. While cell viability testing of all four cell lines exposed to 200 ppm toluene vapor for 1hour did not result in any detectable decrease of cell viability, proteomic analysis displayed minor signs of cellular stress with a majority of modulated proteins. These findings contribute to understanding the complex protein modifications within normally conserved proteins and toluene induced activation of cellular signaling pathways, which will aid in determining initiating mechanisms of brain toxicity from low level doses.

15. SUBJECT TERMS
toluene, *in vitro*, jet fuel, cell viability, proteomics

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PREFACE

Funding for this project was provided through the Aerospace Toxicology Program, which is part of the Aerospace Physiology and Toxicology Initiative in the Air Force Research Laboratory. This research was conducted under contract FA8650-10-2-6062 with the Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF) and Navy work unit number H1104, which is managed by the Naval Medical Research Unit – Dayton (NAMRU-D). The program manager for the HJF contract was David R. Mattie, PhD (711 HPW/RHDJ), who was also the Aerospace Toxicology Program Manager for this project.

The authors would like to acknowledge Dr. Schubert of the Salk Institute for Biological Studies in La Jolla CA, for the generous gift of the HT22 immortalized mouse hippocampal cell line.

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1.0 SUMMARY

Toluene, a volatile organic compound and a component of jet fuel, represents a potential contaminant in aircraft. Effects of toluene exposure include headache or fatigue at 50 to 100 ppm (Zenz *et al.*, 1994), with 75 ppm as the lowest adverse effect level for impairment in performance tests (Ostergaard, 2000). The objective of this study was to investigate the cellular and molecular effects of toluene on murine cell lines, including Cath.a (neuronal cell line), C8-B4 (cerebellar macrophage-derived microglia cell line), CLU199 (embryonic hippocampal hypothalamic cell line), and HT22 (hippocampal cell line).

This study examines: 1) a direct toluene dosing model (various concentrations of toluene in a dimethyl sulfoxide (DMSO) vehicle were added in cell culture medium), and 2) a vapor exposure model (known concentrations of toluene vapor were passed through a glass exposure chamber). To better define the complex signaling response to toluene, these two exposure models are characterized by the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrzolium inner salt] (MTS) cell viability assay, and the preliminary proteomics were performed by tandem mass tag (TMT) liquid chromatography mass spectrometry (LC-MS/MS) quantitative proteomics pathway analysis.

Cell viability testing of direct dosing of 0 to 25 mM toluene in DMSO for 24 hours demonstrated a dose-dependent decrease in cell viability for all cell lines. A preliminary Cytoscape 3.1.0 Reactome FI pathway analysis of Cath.a neuronal cells following direct dosing with 0.2 and 2 mM toluene in DMSO revealed a dose-dependent toxicity increase of modulated protein nodes related to oxidative stress such as energy reserve metabolism, cell-death signaling, reactive oxygen species (ROS) defense, cytoskeletal rearrangement, DNA damage response, targeted protein degradation, and apoptosis.

While cell viability testing of all four cell lines exposed to 200 ppm toluene vapor for 1 hour in the glass chamber did not result in any detectable toluene toxicity by MTS assay, preliminary proteomic analysis displayed minor signs of cellular stress. The majority of modulated proteins were related to cell adhesion, gene expression, ion channel activity, migration, microtubule/microfilament movement, axon guidance/synapse maturation, differentiation, glutamate signal transduction, immune response, and cytoskeletal organization. In addition, a minor number of modulated proteins were related to apoptosis, DNA damage response, and chromatin remodeling with cell-line-specific variations.

These findings contribute to understanding the complex protein modifications within normally conserved proteins and toluene induced activation of cellular signaling pathways. The study aids in determining initiating mechanisms of brain toxicity from low level toluene exposures.

2.0 INTRODUCTION

Toluene (methylbenzene, density 0.866 g/mL, molecular weight 92.13 g/mole), is a clear, colorless, and volatile aromatic hydrocarbon (Merck Index, 1989). It is widely used in commercial products for paints and paints thinner, nail polish, lacquers, and rust inhibitors. It is also a component of jet fuel (1.1 percent in vapor; 0.3 percent in neat JP-8) that has been measured in minute quantities on aircraft (Hester *et al.*, 2011; NEPM, 2003). Since it is used widely in consumer products, its concentration in indoor air may be higher than outdoor.

Critical health effects are central nervous system (CNS) depression and respiratory irritation during short-term exposures. CNS effects can include dizziness, drowsiness, headache, fatigue, and nausea (Echeverria *et al.*, 1989; Ostergaard, 2000). Echeverria *et al.* (1989) reported a dose-dependent response in the number of headaches and complaints of eye irritation when volunteers were exposed to toluene vapor. Acute toluene exposure caused global gene expression changes in rat brain, and a molecular complement to the behavioral and neurophysiological changes was observed (Hester *et al.*, 2011). Chronic and intense abuse of toluene resulted in leukoencephalopathy, a syndrome characterized by dementia, cerebellar ataxia, and cranial neuropathies (Filley *et al.*, 2004). Long-term (chronic) exposure to a time weighted average of toluene exceeding 100 ppm was associated with impaired neuropsychological function (Eller *et al.*, 1999). The objective of this study was to elucidate initial mechanisms of toluene toxicity in neuronal cell lines.

To correlate current *in vivo* toluene exposure data in the rat, an *in vitro* model was investigated using four murine neuronal cell lines: Cath.a transgenic mouse neuronal cell line (ATCC, Manassas VA), C8-B4 mouse cerebellar immortalized macrophage-derived microglia cell line (ATCC), CLU199 mouse embryonic hippocampal hypothalamic cell line (Cedarlane USA, Burlington NC), and HT22 immortalized mouse hippocampal cell line (Salk Institute for Biological Studies, La Jolla CA). Tandem Mass Tag (TMT) liquid chromatography mass spectrometry (LC-MS/MS) proteomics analysis was utilized; this method is uniquely suited for identification and quantitation of large numbers of proteins. The technique is based on labeling individual samples with different isobaric mass tags, which can be differentially detected in a pooled sample setting and allow analysis of up to 10 samples simultaneously. In detail, the tags display the same hydrophobicity and therefore are eluted from the ultra-performance liquid chromatography (UPLC) column at the same time and with the same parent mass. During fragmentation, the label is cleaved and a unique reporter ion is created for each sample that allows for easy relative quantitation between samples. The combining of samples also allows for signal enhancement of some proteins as the signal can be amplified by the larger number of samples.

Following identification and quantitation, the modulated proteins were further analyzed using Cytoscape 3.1.0 (Shannon *et al.*, 2003) to assess the protein pathway modulations that occur following exposure to toluene. This unique approach of broad-spectrum TMT-LC-MS/MS proteomic profiling in combination with Cytoscape 3.1.0 proteome pathway analysis was employed to better define the complex signaling response to toluene.

To date, no comparable study using TMT-LC-MS/MS quantitative proteomics has been conducted with regards to toluene exposure broad-spectrum functional interaction network pathway analysis. Besides giving insight into the complex toluene-related signaling responses, the data can give preliminary information about temporal exposure and dose-response relationships.

3.0 METHODS

3.1 Cell Culture

Four different murine brain cell lines (Table 1) were used in this study to evaluated different cell types and regions found in the brain. All the cell types were maintained in a humidified incubator with 5 percent CO_2 at 37˚C.

Table 1. Cell Lines with Their Growth Conditions

Cell line /vendor	Media w/ Vendor Supplements*	Adherence	Flask type
Cath.a, ATCC	RPMI-1640, ATCC 8% horse serum 4% FBS	mixed	Poly-D-lysine coated
C8-B4, ATCC	DMEM, ATCC 10% FBS 1% Pen Strep	adherent	Tissue culture (TC) treated
HT-22, **	DMEM, ATCC 10% FBS 1% Pen Strep	adherent	TC treated
CLU 199, Cedarlane	DMEM, Sigma 10% FBS 1% Pen Strep	adherent	TC treated

Notes: *horse serum, fetal bovine serum (FBS) and 1% penicillin/streptomycin were from ATCC; **generous gift from Dr. Schubert at Salk Institute, CA USA

3.2 Toluene Direct Dosing in Tissue Culture Media

For the dose-response study, Cath. a, C8-B4, CLU199, and HT22 cells were seeded in 24-well plates at a density of 150 K/well (n = 3). After 24 hours, cells were dosed with toluene (0, 0.75, 1.5, 3, 6, 12, and 24 mM) in dimethyl sulfoxide (DMSO) for 24 hours. Dosing volume was 10 µL.

3.3 Toluene Vapor Exposure in Glass Chamber

Custom-made glass chambers (2.4 L, Figure 1) were used to expose the cells to air and 200 ppm toluene vapor for 1 hour. Lumox® 24-well plates (Sarstedt, Numbrecht, Germany) were employed for glass chamber exposures. Lumox® 24-well plates are manufactured with a polystyrene frame and a 50 µm thick fluorocarbon film bottom; this film is vapor permeable and allows toluene vapor to pass, thereby exposing the cells. For control exposures, the cells were exposed to laboratory air concurrently while the cells dosed with toluene vapor (200 ppm) were in the exposed chamber. Both chambers were placed in a 37 ˚C incubator (Figure 2) during the experiment period.

A schematic of the flow path is shown in the Appendix. Atmosphere distribution testing was performed using unfiltered Pall Mall cigarettes. The smoke patterns showed excellent distribution of the test atmosphere to all areas of the *in vitro* chamber regardless of inlet position.

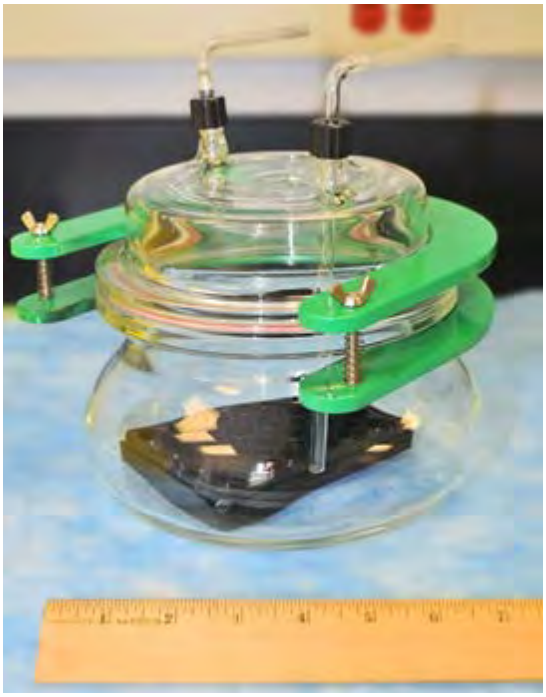


Figure 1. A Custom-Made Glass Chamber with a Lumox® 24-Well Plate. Chamber dimensions are 7 inches wide by 6 inches high.

Figure 2. Two Custom-Made Glass Chambers with 24-Well Plates in the Incubator. One chamber is for the control exposure, and the other for the toluene exposure.

3.4 Cell Viability Testing

Cell viability was assessed for both exposures using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison WI) that is based on metabolic conversion of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to the colored formazan product. The product, soluble in cell culture medium, is produced only by viable proliferating cells. The assay was performed according to manufacturer instructions. In brief, 10 µL of reagent was added to 100 µL of culture medium followed by a 4-hour incubation at 37ºC. Metabolic conversion was measured at 490 nm absorbance. The average absorbance of non-exposed control cells were compared with that of exposed cells. Percent viability was then calculated.

3.5 Quantitative Mass Spectrometry Proteomics Analysis

Cells from both exposures (Sections 3.1 and 3.2) were centrifuged at 500 G for 5 minutes. Supernatant was removed and cell pellets were washed twice with 1 mL phosphate buffered saline (PBS). Cell pellets were lysed in 100 µL of lysis buffer (8M urea, 50 mM triethyl ammonium bicarbonate (TEAB), and 75 mM NaCl), and homogenized with a handheld pestle homogenizer. Protein samples were then processed by the addition of 5 mM tris (2-carboxylethyl) phosphine hydrochloride (TCEP) and 15 mM iodoacetamide (IAA) for reduction and alkylation of cysteines. Processed protein samples were diluted and digested overnight at 37°C with 4 µg of trypsin, followed by quenching with 1 percent formic acid. Samples were

desalted using a C18 peptide trap from Michrom (Auburn CA). The desalted samples were vacufuged and processed by TMT labeling according to the manufacturers' instructions (Applied Biosystems, Life Technologies, Grand Island NY).

Samples were separated into the following analysis groups: 1) CLU199 control, CLU199 experimental, C8-B4 control, C8-B4 experimental, HT22 control, HT22 experimental; and 2) Cath.a control, Cath.a experimental. Samples were then labeled individually with different mass/charge TMT tags and pooled together for comparison. Pooled samples were brought up to 100 µL with water containing 0.1 percent formic acid, and placed in the auto-sampler for injection. Peptides were separated on a Waters BEH C18 capillary column (Milford MA) prior to analysis using a 240-minute increasing gradient of acetonitrile with 0.1 percent formic acid. Following elution from the column, ions were generated using 1.4 kV on a coated capillary tip in a New Objective source (Woburn MA) and entered into the linear trap quadrupole (LTQ)-Orbitrap Velos (Thermo Fisher, San Jose CA). A full scan was taken in the LTQ, followed by data-dependent tandem mass spectrometry (MS/MS) analysis of the top 8 peaks. MS/MS analysis included collision-induced dissociation (CID) in the LTQ for structural information and higher-energy collisional dissociation (HCD) in the Orbitrap for quantitation. To enable low abundance detection, peptide analysis was executed under dynamic exclusion, followed by peptide alignment with a mammalian protein database using the SEQUEST algorithm in Proteome Discoverer 1.3 (Thermo Fisher, San Jose CA). Variable modifications for TMT labeling (N-terminal labeling on amino acid lysine), methionine oxidation, and cysteine carboxymethylation were included in the database search. Data were searched using the SEQUEST algorithm in Proteome Discover 1.3. SEQUEST program analysis included determination of experimental group TMT ratios by division through the average values derived from their controls followed by normalizing group values by division of fold expression values through the median for group comparison.

3.6 ClueGO GO Term Analysis

SEQUEST defined EnsemblProt accession numbers were converted via the web-based Biomart (http://central.biomart.org) conversion tool into WikiGene names prior to analysis by the Cytoscape 3.1.1. built-in application ClueGO2.1.5. Following exclusion of non-modulated proteins (between 0.83 and 1.2 fold expression compared to control), toluene modulated proteins were entered into ClueGO by separating upregulated (above 1.2 fold) and downregulated (below 0.83 fold) proteins. The program algorithms were set to detailed network, and biological process identification was set to significance level of 0.05 to narrow down gene ontology definitions.

3.7 Reactome FI Network Analysis

SEQUEST defined EnsemblProt accession numbers were converted via the web-based Biomart conversion tool into WikiGene names prior to entering the complete WikiGene set into the Cytoscape 3.1.1. built-in application Reactome FI 4.1.1.beta under the 2013 algorithm for "gene sets" using "Fetch FI annotations" for network prediction. Following network prediction and clustering the resulting networks modules were classified by screening for "GO Biological

Process." For comparison of node expression within network clusters a gradient coloring scheme was applied to display upregulated (above 1.2 fold expression, red color) vs. downregulated (below 0.83 fold expression green color) protein nodes. White color nodes represent changes of non-modulated nodes with similar expression to control levels (between 0.83 and 1.2 fold expression).

4.0 RESULTS AND DISCUSSION

4.1 Cell Viability following 24 Hour Direct Dosing to Tissue Culture Media

Cath. a, C8-B4, CLU199, and HT22 cells were exposed to various concentrations of toluene (0 to 25 mM) in DMSO for 24 hours (n=3) by direct dosing. There was a reciprocal relationship between toluene concentration and cell viability (Figure 3). The data support a previous toluene vapor study by Kim *et al.* (2012), who reported a dose-dependent decrease of cell viability in A549 human lung epithelial cells following toluene vapor exposure.

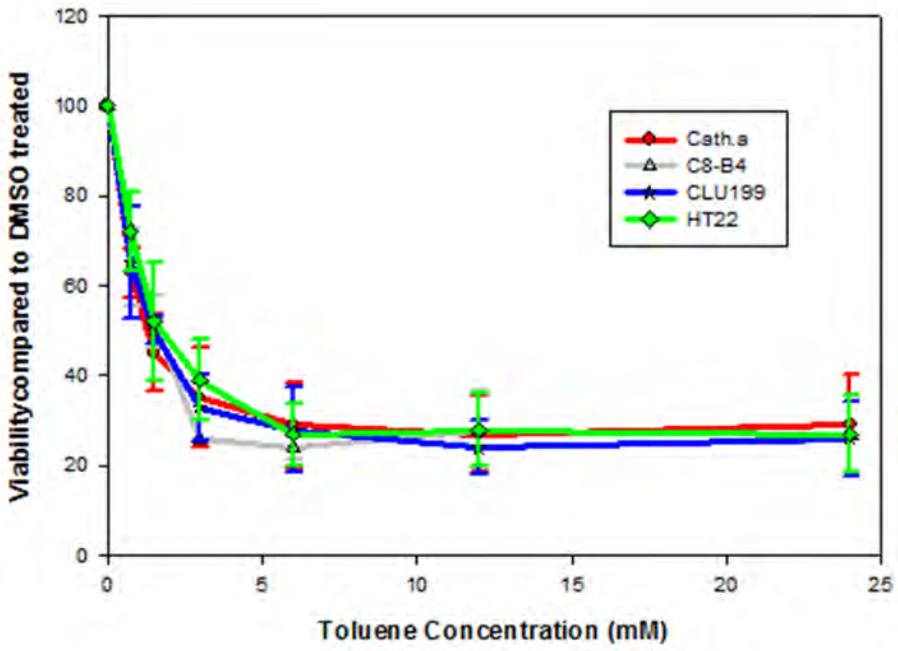


Figure 3. Cell Viability Dose-Response Study Following 24 Hours Direct Dosing of Toluene in the Media

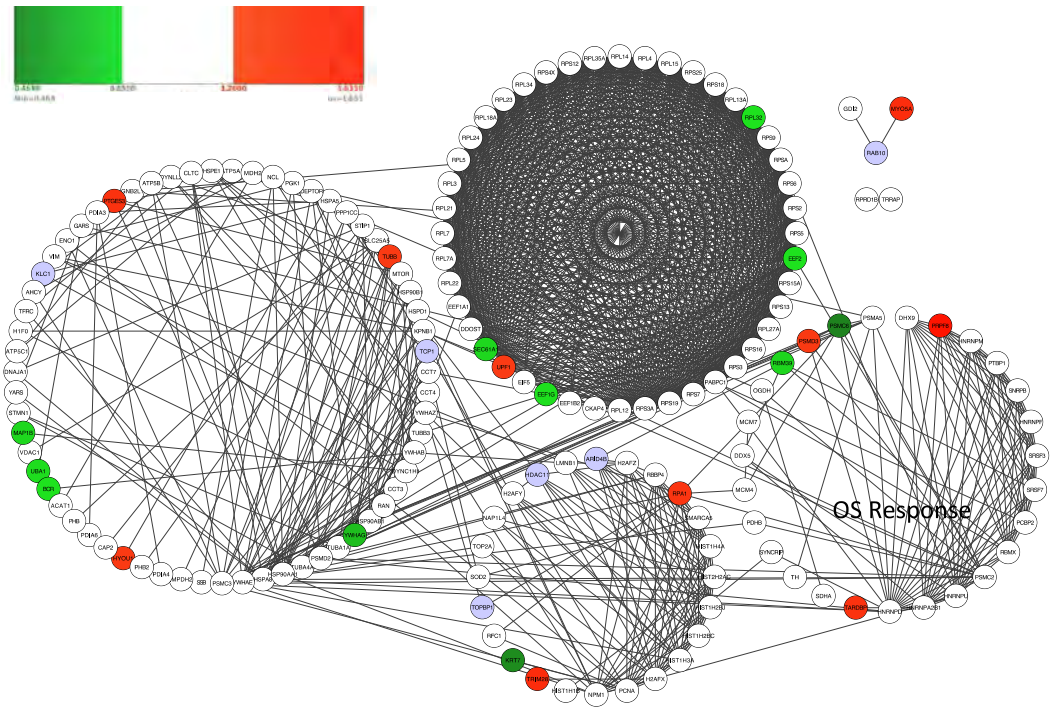
4.2 Cell Viability and Quantitative Proteomics Following One-Hour Vapor Exposure in a Glass Chamber

Cath. a, C8-B4, CLU199, and HT22 cells were exposed to 200 ppm toluene vapor (n=6) or clean air for 1 hour (n=6) in 24-well Lumox^{TM} plates inside glass chambers. No changes in cell viability of different cell lines were observed by MTS assay during the toluene vapor exposure (data not shown).

4.3 Preliminary Quantitative Proteomics Following 24 Hour Direct Dosing to Tissue Culture Media

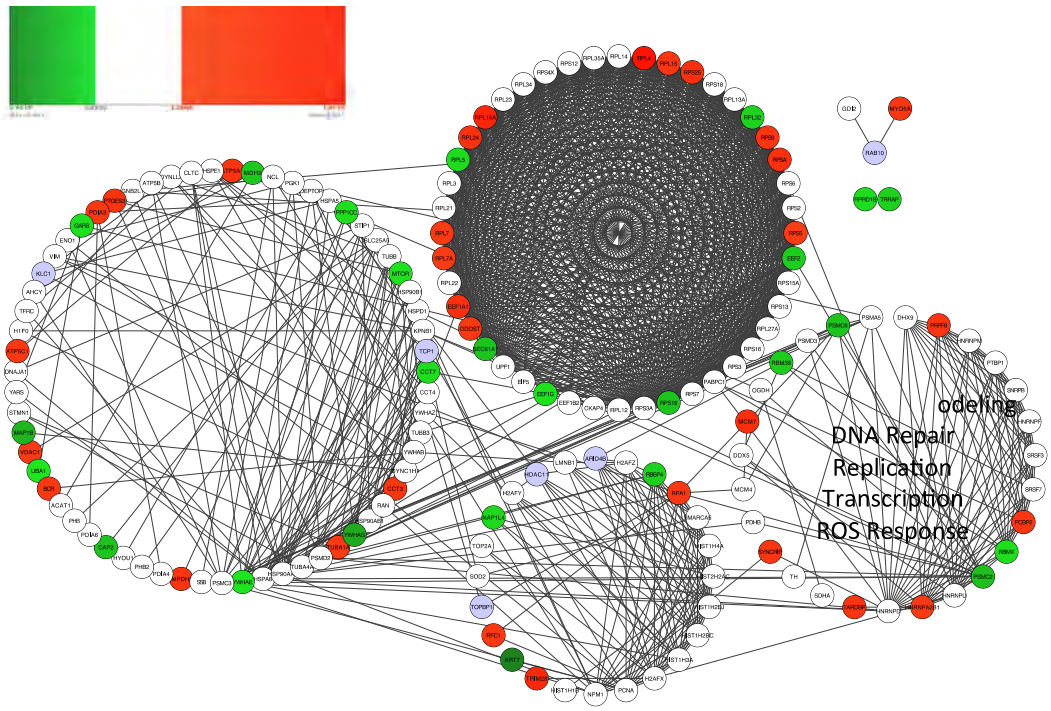
SEQUEST analysis of the MS/MS profiles following 24 hour exposures of Cath.a cells to 0, 0.2, and 2 mM toluene was evaluated using Cytoscape 3.1.0 together with the built-in application Reactome FI 3.0.2beta (Figure 4). Relative peptide quantitation detected 234 proteins that were grouped into 4 main modules with different gene ontology terms. Network modules were characterized by gene ontology (GO biological process) of most abundant nodes contained within the modules. Toluene treatment resulted in a dose-dependent increase of modulated protein nodes related to oxidative stress as characterized by gene ontologies for energy reserve metabolism, cell-death signaling, reactive oxygen species (ROS) defense, cytoskeletal rearrangement, DNA damage response, targeted protein degradation and apoptosis. There was a dose-dependent increase in protein modulation between the DMSO vehicle control, 0.2 mM toluene, and 2 mM toluene.

For comparison of node expression within network clusters, a gradient coloring scheme was applied in Figure 4 to display upregulated (above 1.2 fold expression, red color) vs. downregulated (below 0.83 fold expression green color) protein nodes. White color nodes represent non-modulated nodes with similar expression to control levels (between 0.83 and 1.2 fold expression). Nodes with non-conclusive regulation are displayed in blue.



A.

Figure 4. Cytoscape Reactome Functional Interaction Network following 24 Hours Direct Toluene Exposure. Pathway analyses are shown for Cath.a cells exposed to A) 0, B) 0.2 and C) 2 mM toluene. Colored circles indicate upregulated (above 1.2 fold expression, red), downregulated (below 0.83 fold expression, green), and non-conclusive regulation (blue) protein nodes.



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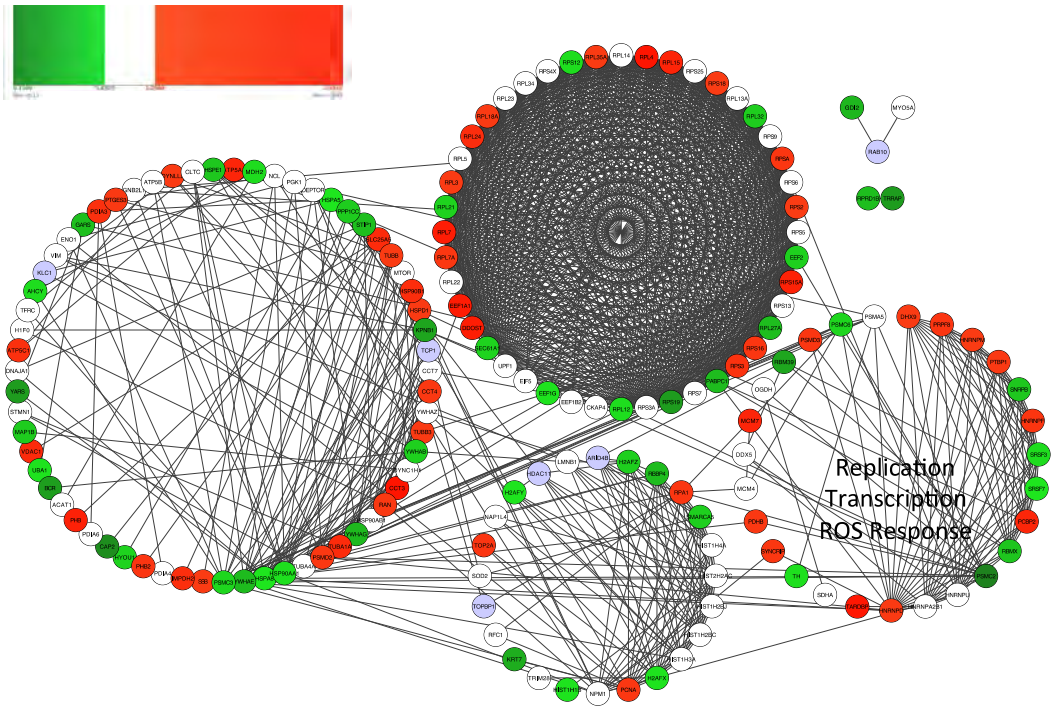


C.

Figure 4 (continued)

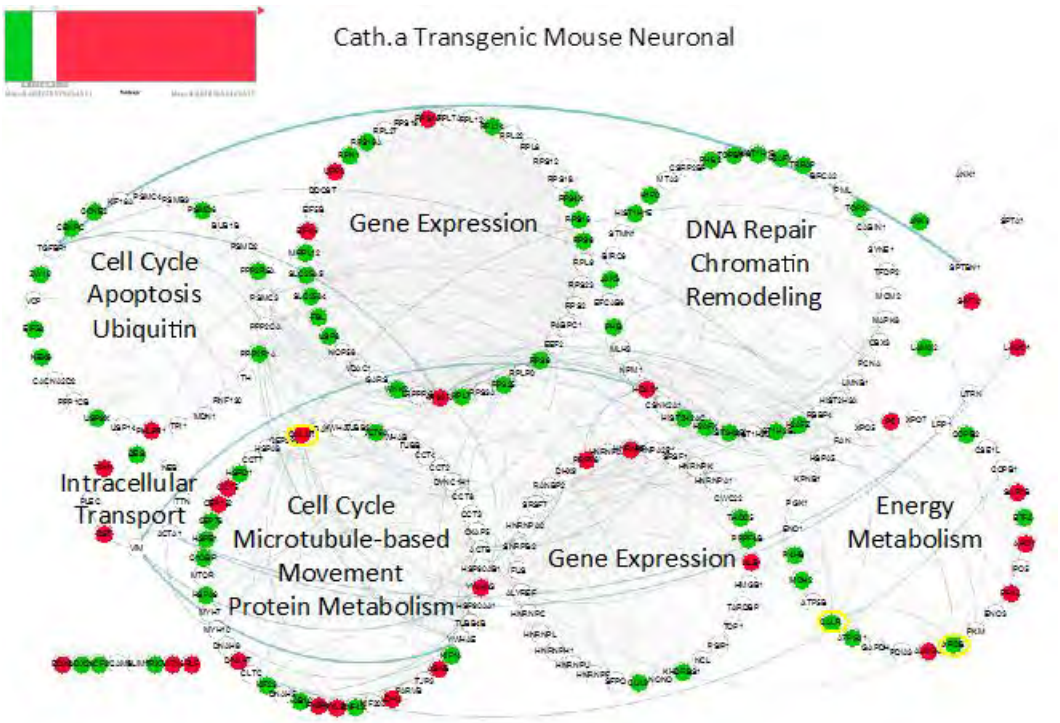
4.4 Quantitative Proteomics Following 1 hour 200 ppm Vapor Toluene Exposure

4.4.1 SEQEST identified MS/MS Proteomic Profile following Vapor Toluene Exposure. SEQUEST relative peptide quantitation of CLU199, C8-B4, and HT22 cell lines resulted in 961 EnsemblProt identifiers of which 908 had a matching WikiGene name identifier. SEQUEST relative peptide quantitation of the Cath.a cell line resulted in 456 EnsemblProt identifiers of which 456 had a matching WikiGene name identifier.

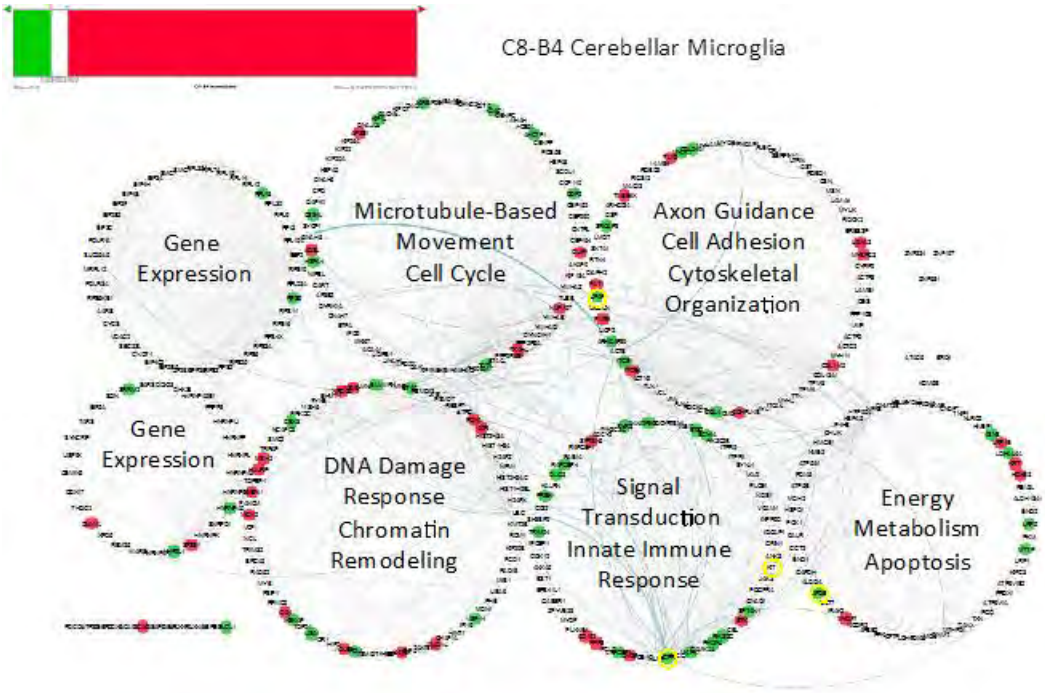
Of the CLU199, C8-B4, and HT22 cell lines identified 908 WikiGene name identifiers between 12.44 to 15.19 percent were downregulated in expression (below 0.83 fold) and between 9.36 to 13.98 percent were upregulated in expression (above 1.2 fold) compared to the control following toluene exposure. Of the Cath.a cell line, 456 WikiGene name identifiers 24.34 percent were downregulated in expression (below 0.83 fold) and 17.32 percent were upregulated in expression (above 1.2 fold) compared to the control following toluene exposure.

4.4.2. Cytoscape Reactome Functional Interaction Network following Vapor Toluene Exposure. The functional interaction network was predicted through the Reactome FI application of Cytoscape 3.1.1. According to the Reactome FI biological network cluster prediction for "GO Biological Process", the 908 WikiGene name identifiers from the CLU199, C8-B4, and HT22 cell lines resulted in 7 network modules larger than 3 nodes (Figure 5). The biggest predicted module was related to GO terms for DNA damage response/chromatin remodeling, followed by modules for microtubule based movement/cell cycle, axon guidance/cell adhesion, energy metabolism, signal transduction, and gene expression. Epidermal growth factor (EGF) receptor signaling seems to play a central role in CLU199 cells where it was upregulated, while it was downregulated in C8-B4 and HT22 cell lines.

For comparison of node expression within network clusters, a gradient coloring scheme was applied in Figure 5 to display upregulated (above 1.2 fold expression, red color) vs. downregulated (below 0.83 fold expression green color) protein nodes. White color nodes represent non-modulated nodes with similar expression to control levels (between 0.83 and 1.2 fold expression). Pathway relevant nodes are labeled in yellow.

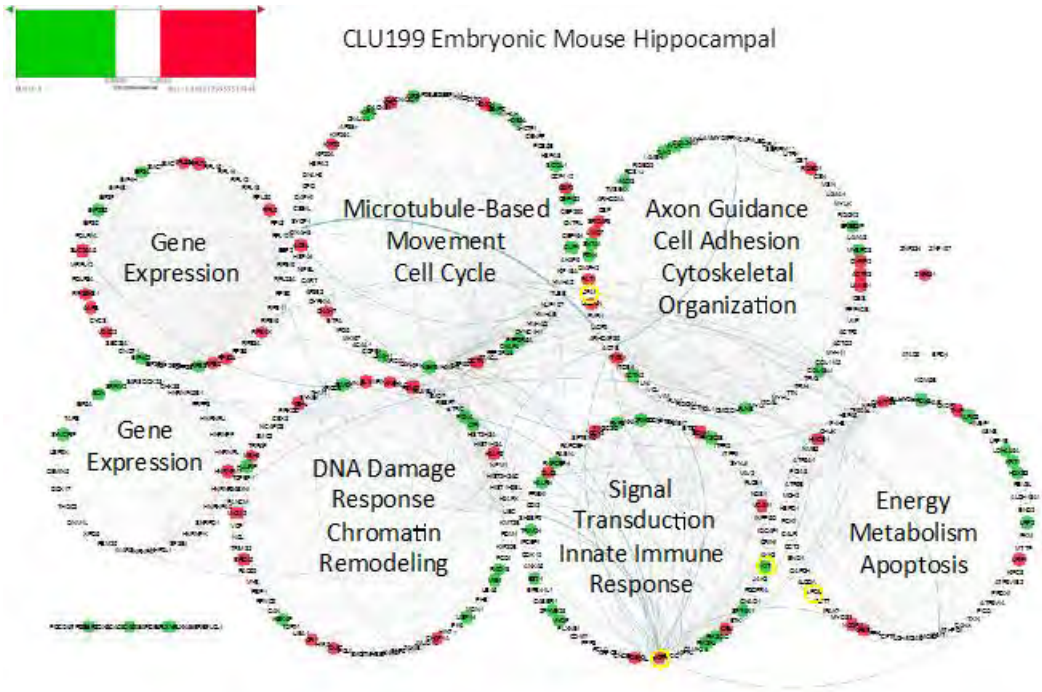


A.



B.

Figure 5. Cytoscape Reactome Functional Interaction Network Following 1 Hour 200 ppm Vapor Toluene Exposure. Cell lines exposed were: A) Cath.a; B) C8-B4; C) CLU199; and D) HT22.



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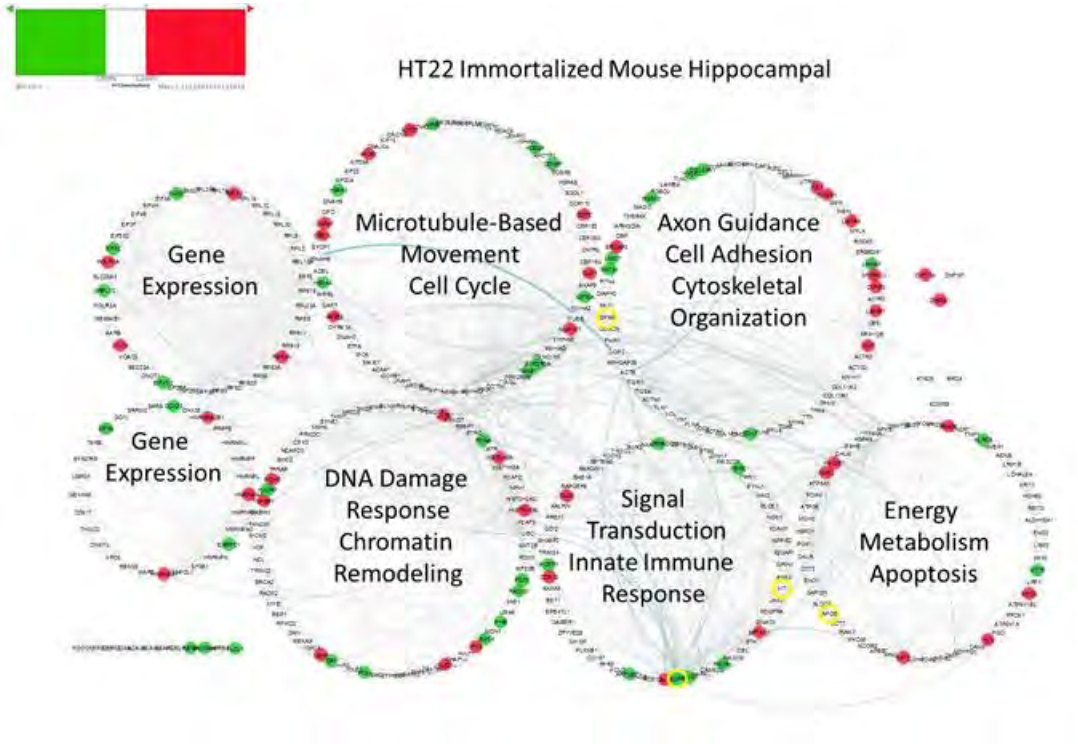
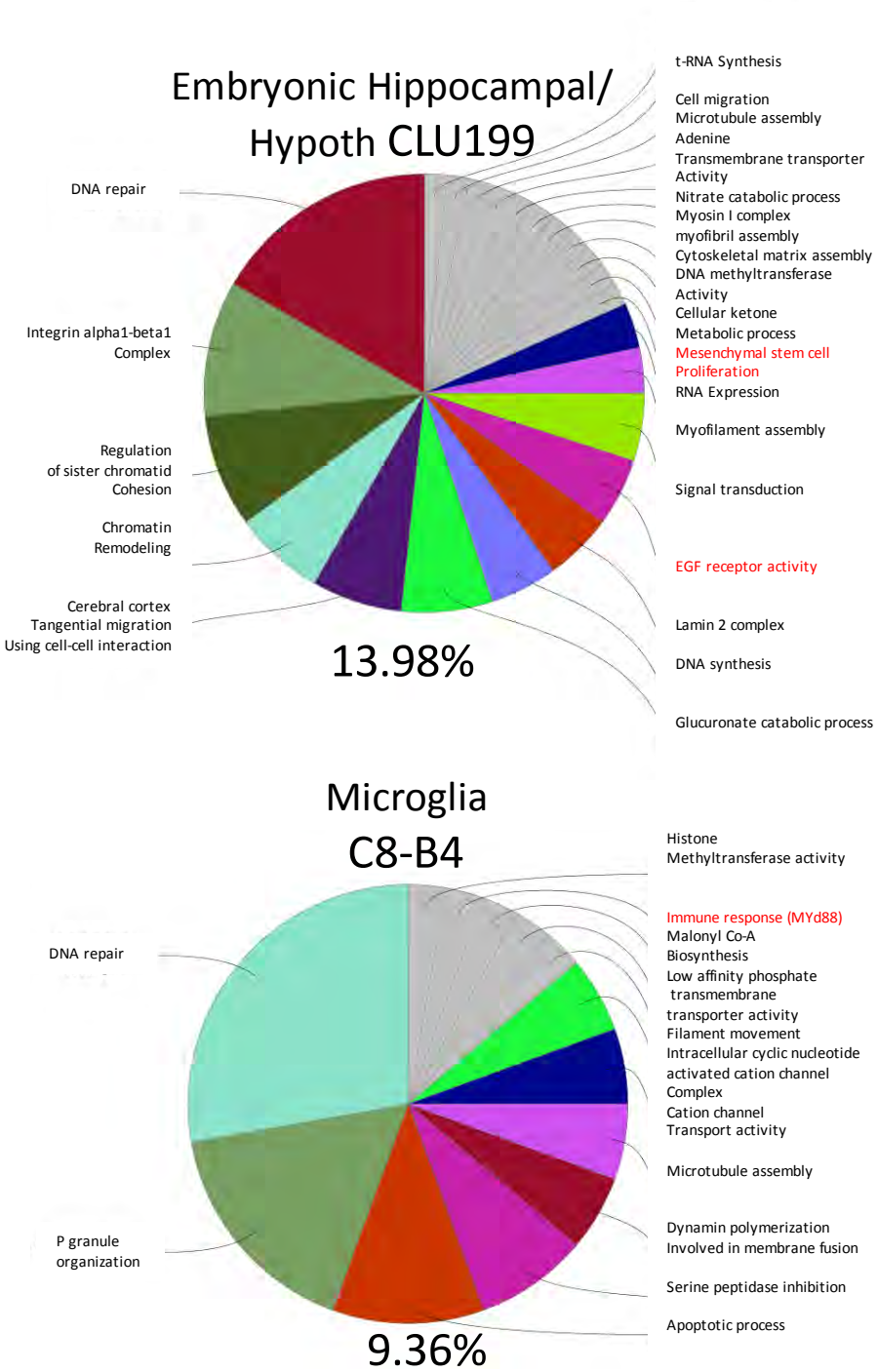


D.

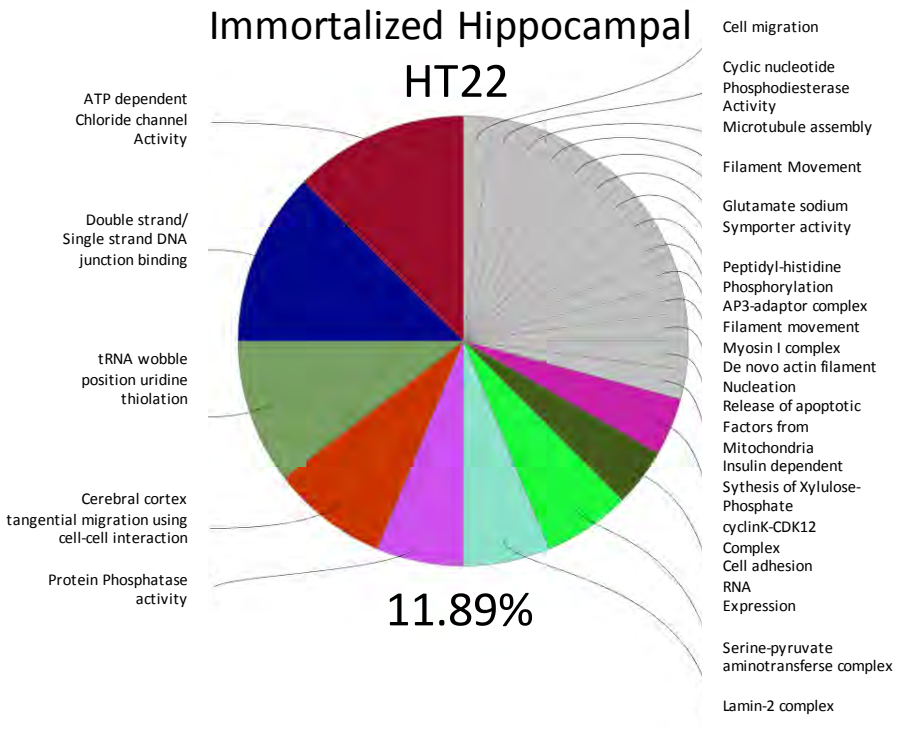
Figure 5 (continued)

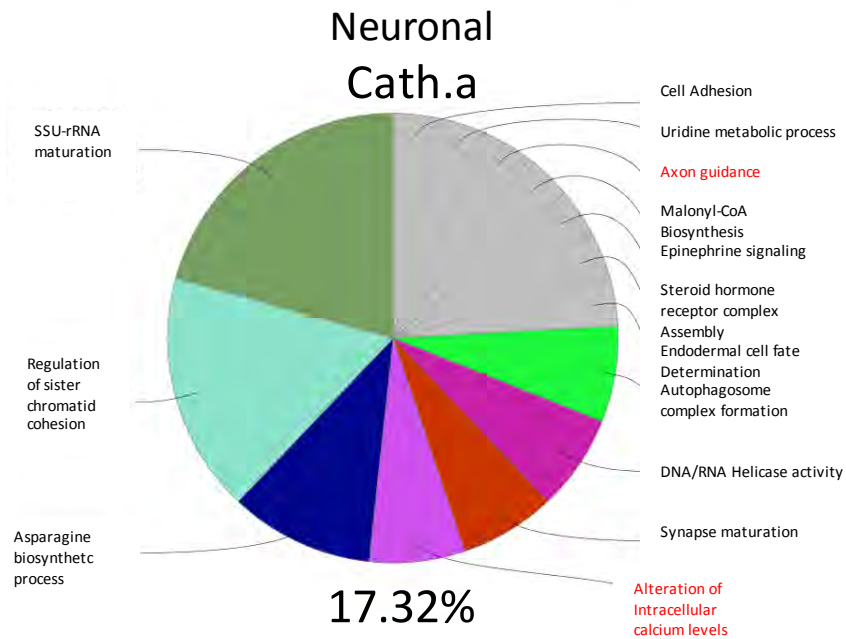
4.4.3 ClueGo Sequential Gene Ontology Proteomic Profile following Vapor Toluene Exposure. Cytoscape 3.1.1 ClueGo sequential gene ontology (GO) analysis for modulated proteins was able to define GO terms for 100 percent of all entered WikiGene name identifiers. GO term analysis of modulated proteins following toluene exposure indicated an upregulation of proteins (Figure 6) for cell adhesion, gene expression and modulation of various ion channel activities in all cell lines. In CLU199, C8-B4, and HT22 cells, GO terms for migration, and microtubule and filament movement were upregulated. HT22 and C8-B4 cells GO terms for apoptosis and DNA damage response were also upregulated. CLU199 demonstrated an upregulation of GO-terms for chromatin remodeling and EGF receptor signaling, similar to Cath.a cells that demonstrated an upregulation of GO terms for axon guidance and synapse maturation. GO-terms of downregulated proteins (Figure 7) in C8-B4, CLU199, and HT22 were defined by EGF receptor signaling, chromatin remodeling, migration, and microtubule and filament movement, together with GO terms for differentiation and DNA repair. Interestingly, GO-terms for glutamate response were downregulated in C8-B4 cells that usually express large amounts of glutamate. Cath.a cells demonstrated a downregulation in GO-terms for adhesion, immune response, and cytoskeletal organization.



A.

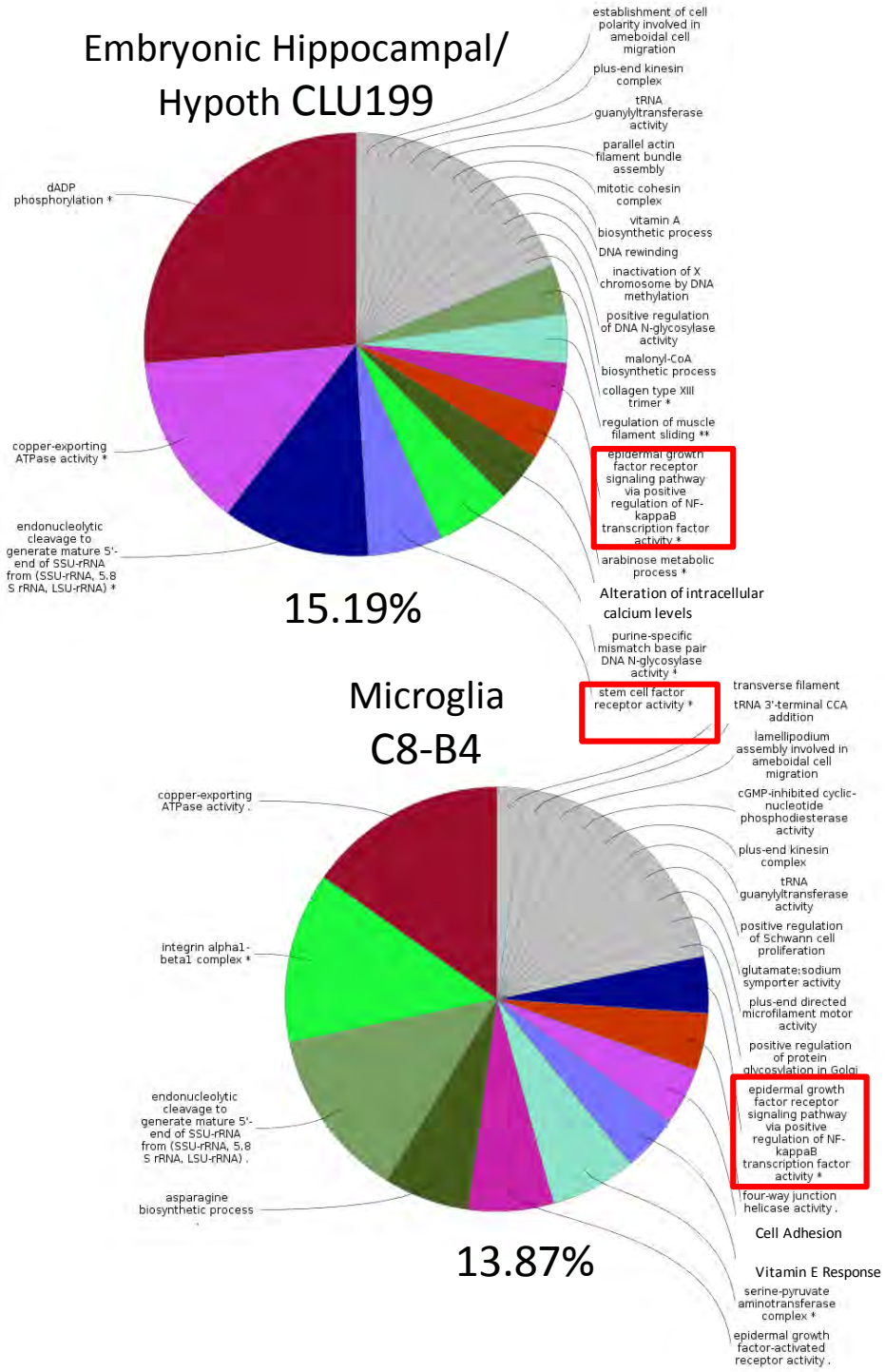
Figure 6. Upregulated ClueGo Sequential Gene Ontology Proteomic Profile Following 200 ppm Toluene for 1 Hour Exposure. Cell lines exposed were A) CLU199, C8-B4, and B) HT22, Cath.a.





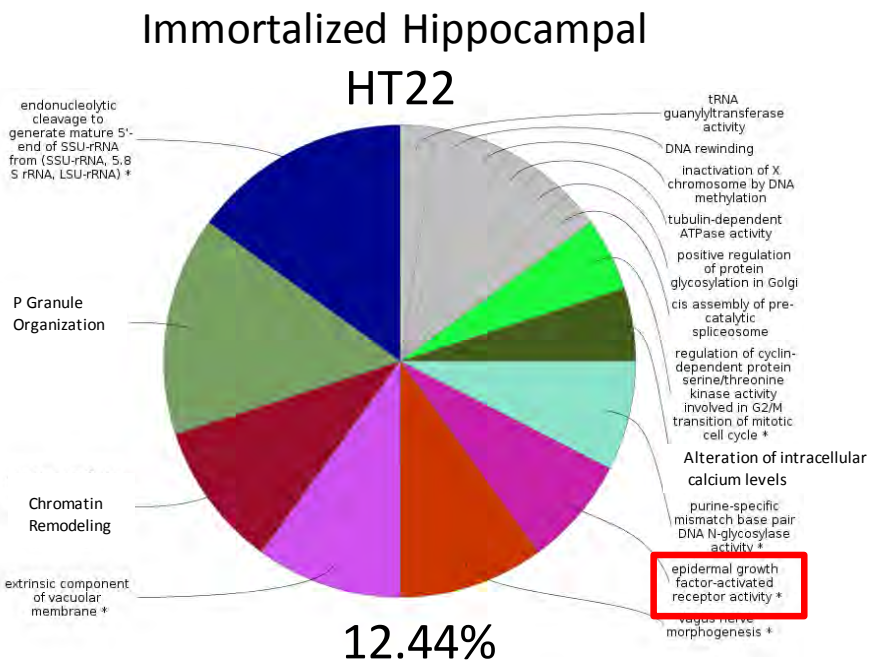
B.

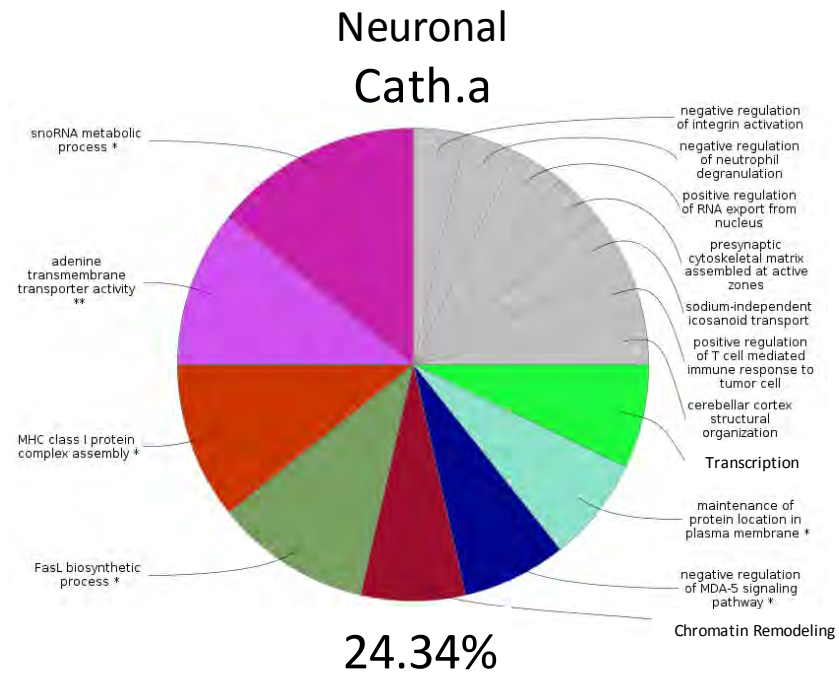
Figure 6 (continued)



A.

Figure 7. Downregulated ClueGo Sequential Gene Ontology Proteomic Profile Following 200ppm Toluene for 1 Hour Exposure. Cell lines exposed were A) CLU199, C8-B4, and B) HT22, Cath.a.





B.

Figure 7 (continued)

4.4.4 Detailed Analysis of the Mass Spectrometry Results. Representative for a transgenic mouse neuronal cell line, Cath.a cells are known to express rat tyrosine hydroxylase, dopamine beta hydroxylase, dopamine, norepinephrine, and synaptophysine. In line with a toluene-dependent toxicity response by interfering with calcium channels (Shafer *et al.*, 2005; Wu *et al.*, 2002), the calcium binding protein calmodulin (CALM, 1.4 fold) was upregulated. In addition, the neuronal structural protein myelin binding protein (MBP) was upregulated 3.09 fold as a sign of cellular structural impairment. Similar to the other cell lines, proteins for modulation of axon guidance and synapse maturation were upregulated. In contrast, a downregulation of calretinin (CALR, 0.78 fold) and apolipoprotein B (apoB, 0.78 fold) was observed together with modulators for adhesion, immune response, and cytoskeletal organization.

According to the CLU199 embryonic mouse hippocampal hypothalamic cell line, characteristic expression of the estrogen receptor alpha and beta, insulin receptor, neuropeptide Y, and brain-derived neurotropic factors (BDNF), toluene induced an upregulation of the EGF receptor which was previously demonstrated to be coupled to the BDNF neurotrophic response (Zadran *et al.*, 2010). In addition, in adherence to the cholinergic properties of this cell line, butyrylcholinesterase (BChE) was upregulated while a downregulation in the ionotropic glutamate receptor GRIK was found. In general, protein modulation was limited to proteins for regulation of apoptosis, DNA repair, chromatin remodeling, cell migration, and microfilament assembly, according to the adhesive characteristics of this cell type.

Similarly in the immortalized mouse hippocampal cell line HT22, characteristic expression of cholinergic markers such as high affinity choline transporter, choline acetyltransferase, vesicular acetylcholine transporter, and muscarinic acetylcholine receptors, an upregulation in BChE was found, while the ionotropic glutamate receptor GRIK was downregulated. In contrast to the CLU199 cells, the adult mouse hippocampal cell line HT22 demonstrated a downregulation of the EGF receptor but matched CLU199 in modulation of apoptotic proteins, DNA repair, chromatin remodeling, migration, and microfilament assembly.

The cerebellar macrophage-derived microglia cells C8-B4 that express large amounts of glutamate demonstrated a distinct response to toluene by displaying a downregulation of the ionotropic glutamate receptor GRIK together with the lipid transport molecule apolipoprotein B (apoB, 0.78 fold) together with a 0.66 fold downregulation in GFAP and the EGF receptor. Previously, these microglia cells were demonstrated to display an excessive calcium-dependent signaling response that is matched by our observation of glutamergic compensatory mechanisms (Langfelder *et al.*, 2015). The general cellular response matched both hippocampal cell lines in displaying modulations of apoptotic proteins, DNA repair, chromatin remodeling, migration, and microfilament assembly. In addition, in line with its microglia characteristics, changes in inflammatory mediators or cytokine response were detected in this cell line. A summary of the toluene vapor exposure study is displayed in Table 2.

Table 2. Summary Proteomics Analysis of Four Cell Lines Exposed to Toluene Vapor at 200 ppm for 1 Hour in a Custom-Made Glass Chamber

Cell Line	Express	Upregulated	Downregulated	Modulated
Cath.a	rat tyrosine hydroxylase, dopamine beta hydroxylase, dopamine, norepinephrine, synaptophysine, SV40 T-ag	**CALM, MBP, EGFR signaling, axon giudance, synapse maturation	CALR, APOB, adhesion, immune response, cytoskeletal organization	----------
C8-B4	large amounts of glutamate	----------	ionotropic glutamate receptor GRIK, APOB, GFAP, EGFR	Apoptosis, DNA Repair, chromatin remodeling, migration, microfilament assembly
CLU199	estrogen receptor alpha and beta, insulin receptor, neuropeptide Y, BDNF, and SV40 T-ag	EGFR, BChE	ionotropic glutamate receptor GRIK	Apoptosis, DNA Repair, chromatin remodeling, migration, microfilament assembly
HT22	cholinergic markers such as high affinity choline transporter, choline, acetyltransferase, vesicular acetylcholine transporter, and muscarinic acetylcholine receptors	BChE	ionotropic glutamate receptor GRIK, EGFR	Apoptosis DNA repair, chromatin remodeling, migration, microfilament assembly

Notes: **CALM - calmodulin; MBP - myelin basic protein; EGFR - epidermal growth factor receptor; CALR - calreticulin protein; APOB - apolipoprotein B; BDNF - brain-derived neurotrophic factor; BChE - butyrylcholinesterase

5.0 CONCLUSIONS

Direct toluene exposure showed a dose-response decrease of MTS cell viabilities in all cell lines. Preliminary quantitative proteomic analysis of cell lines directly exposed to toluene revealed that there was a vehicle toxicity effect of DMSO by direct dosing. Exposure of the cell lines to toluene vapor did not demonstrate any changes in MTS viability assessment.

However, preliminary quantitative proteomic analysis discovered cell-line specific protein modulation following toluene vapor exposure. The results of vapor exposure support the use of vapor permeable Lumox® microplates as suitable culture vessels for toluene in the glass chamber. The quantitative proteomics identified multiple pathway modulations following toluene vapor exposure, including cell adhesion, DNA repair, chromatin remodeling, migration, cytoskeletal organization, and EGF receptor signaling. EGF receptor signaling seems to play a central role in the embryonic mouse hippocampal hypothalamic CLU199 cells, where it was upregulated, while it was downregulated in the adult immortalized hippocampal cell line HT22 and the C8-B4 macrophage-derived microglia cell lines. The upregulation of the EGF receptor in embryonic CLU199 cells might be indicative of a higher regenerative potential of this cell line compared to the adult immortalized cell lines that might be more prone to tumorigenesis and lack of functional trophic repair mechanisms (Siddiqui et al, 2012). Yet the adult cell lines are a necessary component of the cell culture model since they express most of the components of adult brain cells. It remains to be determined if neuronal stem cell co-culture models can better simulate the adult brain versus the separate culture of cell type-specific immortalized cell lines.

In future, replicates of proteomic studies would be beneficial on direct dosing and toluene vapor exposure in the glass chamber. No studies on proteomics of toluene dosing directly or vapor exposure were reported in the literature.

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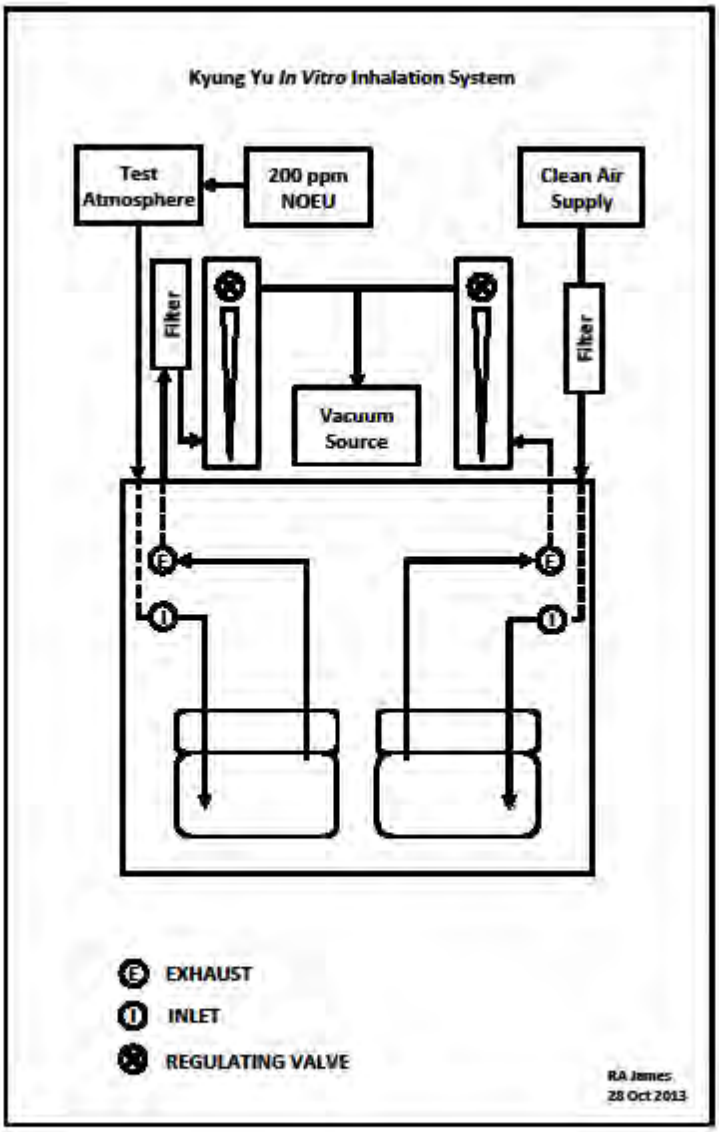
APPENDIX. FLOW PATH OF *IN VITRO* INHALATION SYSTEM



LIST OF ACRONYMS

apoB	apolipoprotein B
BChE	butyrylcholinesterase
BDNF	brain-derived neurotrophic factor
CALM	calmodulin
CALR	calreticulin
CID	collision induced dissociation
CNS	central nervous system
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
FBS	fetal bovine serum
GO	gene ontology
HCD	higher-energy collisional dissociation
IAA	iodoacetate
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LTQ	linear trap quadrupole (mass spectrometer)
MBP	myelin basic protein
MS/MS	tandem mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PBS	phosphate buffered saline
ROS	reactive oxygen species
TC	tissue culture
TCEP	tris (2-carboxylethyl) phosphine hydrochloride
TEAB	triethyl ammonium bicarbonate
TMT	tandem mass tags
UPLC	ultra performance liquid chromatography